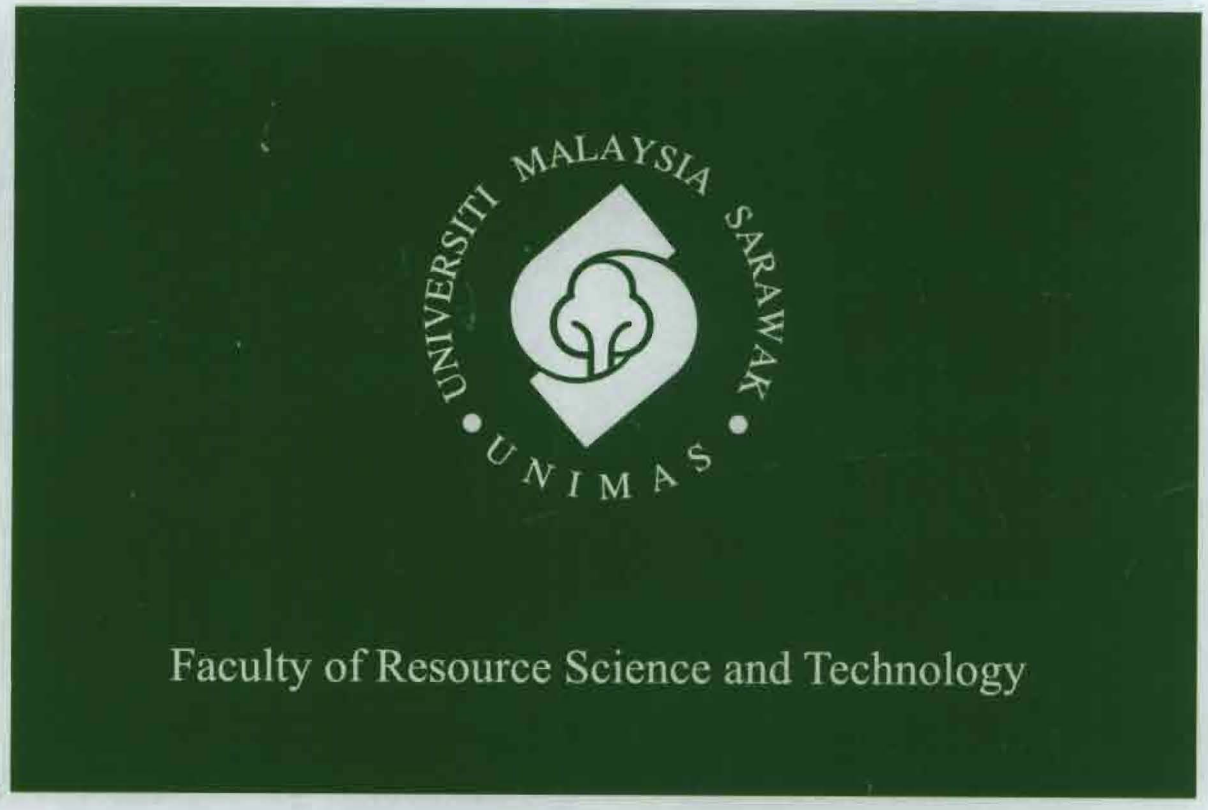

ANTIBIOSIS ACTIVITIES AGAINST *BURKHOLDERIA PSEUDOMALLEI* BY OTHER *BURKHOLDERIA* SPECIES

Nur Ezzah Binti Sainei

(35096)

Bachelor of Science with Honours
(Resource Biotechnology)
2015

UNIVERSITI MALAYSIA SARAWAK

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Final Year Project Report ☑

Masters ☐

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Antibiosis Activities against *Burkholderia pseudomallei* by Other *Burkholderia* Species

Nur Ezzah binti Sainei

This project is submitted in partial fulfillment of the requirements for the Degree of Bachelor of Science with Honours (Resource Biotechnology)

Supervisor: Dr. Yuwana Podin
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DECLARATION

"I hereby declare that this dissertation entitled **'Antibiosis Activities against *Burkholderia pseudomallei* by Other *Burkholderia* Species'** is based on my original work and research, except for quotation and citation, which has been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UNIMAS or any other institution."

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LIST OF ABBREVIATIONS

ASH	Ashdown's selective agar
BCC	*Burkholderia cepacia-complex*
CDC	Centers for Disease Control and Prevention
CF	Cystic fibrosis
CFS	Cell free supernatant
CFU	Colony-forming unit
GEN^r	Gentamicin resistance
GEN^s	Gentamicin sensitive
HCl	Hydrochloric acid
HMW	High-molecular weight
LB	Luria Bertani
MDR	Multidrug resistance
MH	Mueller Hinton
MLST	Multi-locus sequence typing
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NT	Northern Territory
rRNA	Ribosomal ribonucleic acid
ST	Sequence type
UHQ	Ultra high quality
VNTR	Variable-number of tandem repeat
ZOI	Zone of inhibition

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Antibiosis Activities against *Burkholderia pseudomallei* by Other *Burkholderia* Species

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ABSTRACT

Burkholderia pseudomallei is known as a significant causative agent of melioidosis and a versatile species of bacteria which inhabit diverse ecological niches including soil and water sources. By studying and understanding the nature of antibiosis activities against *B. pseudomallei* by other antagonistic *Burkholderia* species, *Burkholderia* spp. that are potentially capable of inhibiting the growth of *B. pseudomallei* are determined to serve as possible biocontrol for the widespread of melioidosis. In this study, certain *Burkholderia* spp. such as *Burkholderia cepacia-complex* and *Burkholderia ubonensis* displayed strong inhibition against *B. pseudomallei*. Bacteriocins in a cell free state (CFS) isolated from *Burkholderia* spp. were found to inhibit *B. pseudomallei* as well. Further characterization of the bacteriocins through various heat treatments indicated that the stability of bacteriocins to heat treatments varies greatly depending on strain-type and exposure to alkaline conditions substantially enhanced the inhibitory activities of the bacteriocins. Subjection to trypsin digestion and freezing showed that the bacteriocins contained both protease-sensitive and phage-like compounds.

Keywords: Antibiosis, bacteriocin, *Burkholderia pseudomallei*, melioidosis, zone of inhibition.

ABSTRAK

Burkholderia pseudomallei dikenali sebagai agen penyebab melioidosis yang ketara dan juga spesies serba boleh yang mendiami pelbagai ceruk ekologi termasuklah tanah dan sumber air. Dengan mempelajari dan memahami sifat aktiviti antibiosis terhadap *B. pseudomallei* oleh spesies *Burkholderia* lain yang bersifat antagonistik, spesies *Burkholderia* yang berpotensi untuk menghalang pertumbuhan *B. pseudomallei* dapat ditentukan untuk dijadikan sebagai pengawalan biologi kepada melioidosis yang berleluasa. Dalam kajian ini, beberapa spesies *Burkholderia* seperti *Burkholderia cepacia-complex* dan *Burkholderia ubonensis* menunjukkan perencatan yang kuat terhadap *B. pseudomallei*. Bakteriosin yang diasingkan dalam keadaan sel bebas (CFS) daripada spesies *Burkholderia* didapati juga merencat pertumbuhan *B. pseudomallei*. Pencirian lanjut mengenai bakteriosin melalui pelbagai rawatan haba menunjukkan bahawa kestabilan bakteriosin adalah amat berbeza bergantung kepada jenis strain serta pendedahan terhadap keadaan beralkali mampu meningkatkan perencatan aktiviti bakteriosin dengan ketara sekali. Pendedahan kepada penghadaman tripsin dan pembekuan menunjukkan bahawa bakteriosin mengandungi sebatian yang sensitif kepada enzim protease dan juga sebatian yang mirip seperti faj.

Kata kunci: Antibiosis, bakteriosin, *Burkholderia pseudomallei*, melioidosis, zon perencatan.

1.0. INTRODUCTION

Burkholderia pseudomallei is a bacterium that inhabits a variety of natural environments including water and soil. The environmental prevalence of the microorganism is significantly correlated with the emergence of an endemic tropical illness known as melioidosis (Marshall *et al.*, 2010). Distribution of *B. pseudomallei* in diverse ecological niches is extensive yet overtly uneven due to the dispersal of other *Burkholderia* species within the same niche (Trakulsomboon *et al.*, 1999). The presence of different *Burkholderia* spp. establishes a co-existence which allows the occurrence of antibiosis activities of certain *Burkholderia* spp. against *B. pseudomallei.*

B. pseudomallei is generally resistant to gentamicin which is the unique hallmark of the bacterium (Podin *et al.*, 2013). Hence, Ashdown's agar is a suitable medium to grow *B. pseudomallei* as it contains the aforementioned aminoglycoside (Ashdown, 1979). Nevertheless, gentamicin-sensitive *B. pseudomallei* (GEN^s) strains had been isolated and selected for the observation of the microbial antibiosis against the strain by other *Burkholderia* spp. This strain is susceptible to gentamicin and unable to grow on the surface of original Ashdown's media (Podin *et al.*, 2013). Thus, the component of gentamicin is replaced by colistin which does not affect the growth of both GEN^r and GEN^s *B. pseudomallei* strains (Podin *et al.*, 2013).

GEN^s *B. pseudomallei* strain is different from GEN^r *B. pseudomallei* strain due to its incapability to be resistant to gentamicin and a few other drugs because of the mutation within its multidrug efflux system (Podin *et al.*, 2013). The involvement of GEN^s *B.*

pseudomallei in this project is to investigate whether the result of antibiosis activity inflicted on GEN^r *B. pseudomallei* is the same with GEN^s *B. pseudomallei* and whether the mutation within the multidrug efflux system is able to influence the antibiosis activity that is imposed on the strain.

This final year project is carried out to identify *Burkholderia* spp. which are potentially capable of suppressing the growth of *B. pseudomallei* and to study the degree of their inhibitory capabilities by observing the presence of zone of inhibition (ZOI) which appears around the antagonistic colonies on the lawn of *B. pseudomallei.* The identification of these *Burkholderia* spp. may serve as possible future biological control for the widespread of melioidosis caused by *B. pseudomallei.* In addition, further characterization of the antibiosis compounds produced by *Burkholderia* spp. which were less characterized by previous studies may provide a deeper understanding to the fundamental characteristics of the compounds and reveal novel discovery that is related to the less investigated topic of the antibiosis activities against *B. pseudomallei* by other *Burkholderia* spp. Hence, the objectives of this final year project are:

1. To identify *Burkholderia* spp. which are potentially capable of producing antibiosis activities against *B. pseudomallei*;
2. To determine which isolated antibiosis compounds from *Burkholderia* spp. possess the ability to inhibit the growth of *B. pseudomallei*;
3. To observe whether different temperatures and pH conditions are able to influence the stability of the isolated antibiosis compounds;
4. To detect the presence of protease-sensitive and phage-like antibiosis compounds through exposure to trypsin digestion and freezing.

2.0. LITERATURE REVIEWS

2.1. *Burkholderia pseudomallei*

2.1.1. Characteristics

B. pseudomallei is categorized under the genus *Burkholderia* which was established by Yabuchi *et al.* (1992) along with other former members of rRNA homology group II pseudomonads (Yabuchi *et al.*, 1992; Coenye & Vandamme, 2003; Inglis & Sagripanti, 2006). The morphological appearance of *B. pseudomallei* is described in the form of a lean, vacuolated bacillus and often expounded to appear in the shape of a "safety pin" through bipolar staining (Cheng & Currie, 2005). Furthermore, the bacterium is characterized as a facultative anaerobe which possesses the characteristics of being a gram-negative, oxidase-negative, saprophytic bacillus that is capable of motility (Brett & Woods, 2000; Inglis & Sagripanti, 2006).

The morphological appearance of *B. pseudomallei* colonies when cultured on selective agars display various morphologies from the initial form of smooth colonies to dry and wrinkled colonies on further incubation up to several days (Cheng & Currie, 2005; Inglis & Sagripanti, 2006; Chantratita *et al.*, 2007). Nonetheless, the species morphological appearance is greatly dependent on the type of strains and the constituents of the solid media. Certain strains display smooth colonies on the first few days of culture and later transform into dry and wrinkled forms as the days progressed (Inglis & Sagripanti, 2006). However, some other strains persist in the form of apparent mucoid (Inglis & Sagripanti,

2006). In addition, solid media which contain especially glycerol typically exhibit strains of *B. pseudomallei* colonies that have no wrinkling effect (Inglis & Sagripanti, 2006).

Strain-types and media constituents are not the only factors that contribute to the diverse morphotypes exhibited by *B. pseudomallei* sp. The versatile characteristic of *B. pseudomallei* to survive and adapt in a wide range of conditions allows the microorganism to be exposed to short-term evolution as studied on the clinical *B. pseudomallei* samples isolated from melioidosis-infected patients as the disease progressed (Price *et al.*, 2010). Such versatility is due to the presence of an abnormal quantity of variable-number of tandem repeats (VNTRs) within the genome of *B. pseudomallei* which have the tendency to mutate over a short duration of time (Price *et al.*, 2010). Frequent occurrences of mutation in VNTRs of *B. pseudomallei* can alter the expression of the bacterial surface determinants as well as the morphological appearance of the bacterial colonies in order to assist the survival of the microorganism both in environment and in vivo (Chantratita *et al.*, 2007).

The versatility of *B. pseudomallei* allows the microorganism to adapt to diverse ecological niches from damp soil and stagnant water sources in tropical and subtropical countries to the internal organs of animals and humans (Inglis & Sagripanti, 2006; Kaestli *et al.*, 2009; Goodyear *et al.*, 2013). According to Cheng and Currie (2005), *B. pseudomallei* is able to live in hostile environmental settings for more than 70 days and up to 10 years which encompass acidic environments, prolonged nutrient scarcity, wide range of temperature, presence of detergent and antiseptic solutions as well as dehydration (where water content in soil is only less than 10%). The exposure to the various rough conditions may also

improve the selective advantage of the bacterial growth of *B. pseudomallei* (Cheng & Currie, 2005).

B. pseudomallei is generally resistant to gentamicin and other antibiotics which is the unique distinctive feature of the species. Nevertheless, recent studies discovered that there are other strains of *B. pseudomallei* that are specifically susceptible to gentamicin which are found in certain part of the melioidosis-endemic regions such as Sarawak, Malaysia (Podin *et al.*, 2013).

2.1.1.1. Gentamicin-resistant *B. pseudomallei* (GEN^r)

B. pseudomallei is naturally resistant to many types of antibiotics including ureidopenicillins, carbapenems, chloramphenicol, trimethoprim-sulfamethoxazole, tetracyclines, amoxicillin-clavulanate, and third-generation cephalosporins (Ryan *et al.*, 2012). The ability to be resistant to gentamicin is particularly a specific characteristic of *B. pseudomallei* which is the reason why gentamicin is a significant selective component in Ashdown's selective medium used to diagnose melioidosis, a disease that is endemic to tropical regions (Podin *et al*, 2013).

According to the study conducted by Moore *et al.* (1999), the main contributor to the resistance of *B. pseudomallei* to macrolides and aminoglycosides is a multidrug efflux system known as AmrAB-OprA within the microorganism. Multidrug efflux system is common in bacterial pathogens which are capable of establishing multidrug resistance (MRD) by tolerating to lethal doses of many types of drugs that are specifically functioned

to eliminate non-resistant strains (Sun *et al.*, 2014). The bacterial efflux system works by expelling a wide range of antibiotics out of the bacterium through drug extrusion as well as reducing the concentration of intracellular antibiotics and promoting accumulation of mutation within the cell (Sun *et al.*, 2014). Based on the study carried out by Chan *et al.* (2004), there is another efflux system that functions similarly to *B. pseudomallei* AmrAB-OprA pump which is the BpeAB-OprM pump. However, BpeAB-OprM pump differs completely from AmrAB-OprA in terms of their amino acid sequences, chromosomal locations, and most importantly, the specificities to different substrates (Chan *et al.*, 2004).

2.1.1.2. Gentamicin-sensitive *B. pseudomallei* (GEN^S)

According to a study conducted by Podin *et al.* (2013), 86% of *B. pseudomallei* clinical samples isolated from Sarawak were identified to be susceptible to gentamicin. Such susceptibility was found to be originated from a non-synonymous mutation of an important component known as *amrB* within the AmrAB-OprA (Podin *et al.*, 2013). The mutation involves substitution of a threonine to arginine within the highly conserved region of the amino acid sequences (Podin *et al.*, 2013). The study on the effect of mutation on the AmrAB-OprA was also conducted by Moore *et al.* (1999) whereby *B. pseudomallei* of *amr* deletion strain was constructed and was discovered to be hypersusceptible to macrolides and aminoglucosides.

The study carried out by Podin *et al.* (2013) shows that a substantially large amount of GEN^S *B. pseudomallei* was discovered in Sarawak, a part of the Malaysian Borneo which is an extremely rare occurence in other part of the endemic regions of melioidosis. The

distribution of GEN^{s} *B. pseudomallei* encompasses a large area of 60,000 km^2 comprising Kapit, Sibu, and Bintulu (Podin *et al.*, 2013). Since there are *B. pseudomallei* (GEN^{s}) isolates from Sarawak that were used in this project, colistin-containing Ashdown's selective medium was used instead of gentamicin as a selective agent.

2.1.2. Epidemiology

B. pseudomallei is the main etiological agent of melioidosis, an emerging illness which is endemic to northern Australia and southeast Asia correlated with other tropical and subtropical countries within the latitude of 20°N and 20°S (Godoy *et al.*, 2003; Cheng & Currie, 2005; Kaestli *et al.*, 2009). The endemic regions encompass Malaysia, Indonesia, Thailand, Vietnam, Laos, Singapore, southern China, and Northern Territory (NT) of Australia (Cheng & Currie, 2005). Due to the worldwide infection with no available licensed vaccines or proper medical therapies, *B. pseudomallei* is labeled as category B organism by CDC for its possible potential as a biowarfare agent (Thibault *et al.*, 2004; Massey *et al.*, 2014).

The first case of melioidosis was reported in Australia in 1949 from a disease outbreak on sheep followed by human-related cases in 1950 and later the cases in NT, Australia in 1960 (Cheng & Currie, 2005). The late discovery in NT indicated that the colonization of *B. pseudomallei* in Australia may have begun from Southeast Asia (Cheng & Currie, 2005). The highest number of cases of melioidosis-infected patients was documented in Ubon Ratchathani, northeastern Thailand whereby 20% of community-acquired septicemia led to a significant number of mortality (Cheng & Currie, 2005; Kaestli *et al.*, 2009; Galyov *et*

al., 2010). The melioidosis-related cases reported outside the endemic regions majorly involve sporadic distribution of the disease through travelling humans from the highly endemic countries to other countries (Chen *et al.*, 2003; Cheng & Currie, 2005).

The geographical distribution of *B. pseudomallei* which seems to favor the tropical regions close to the equator may be influenced by temperature, water content and pH condition of the soil, as well as salinity. According to the study by Chen *et al.* (2003), the growth of *B. pseudomallei* highly favors warm and hot climates within the tropical countries based on the in vitro study of the microorganisms in soil media at optimum temperature ranging from 37 °C to 42 °C. Nevertheless, *B. pseudomallei* also discovered to be able to survive at 4 °C indicating that melioidosis can also occur in countries that experience winter seasons. *B. pseudomallei* also seems to prefer soil pH of 6.5 to 7.5 with low salinity, 15% water content and high nutritional contents which is why regions correlated with paddy fields have high prevalence of melioidosis (Chen *et al.*, 2003). In addition, high incidence of melioidosis also corresponds to extreme weather conditions such as monsoon seasons and flood which increase the spatial distribution of *B. pseudomallei* from one place to another (Cheng & Currie, 2005; Kaestli *et al.*, 2008).

2.1.3. Pathogenicity

B. pseudomallei can be both epizootic and zoonotic whereby not only it can spread among animal population, the pathogen can also be transmitted from infected animals to humans as occurred in the 1970 outbreak in a Paris zoo, ensuing to the distribution of the disease to other zoos as well as a number of fatalities involving humans and animals (Cheng &

Currie, 2005). In general, *B. pseudomallei* can infect human population via exposure of wounded skin to contaminated soil and water sources, direct inoculation on the skin, ingestion as well as inhalation of the aerosolized microorganisms (Limmathurotsakul *et al.*, 2010; Massey *et al.*, 2014).

B. pseudomallei infections manifest in the form of abscesses in internal organs and in severe cases, septic shock and acute pneumonia (Galyov *et al.*, 2010). In chronic cases, the illness can lead to fatalities. The bacterium is also considered as opportunistic pathogen as found in a number of records that stated the emerging melioidosis is highly correlated to the patients with pre-existing abnormalities such as diabetes mellitus, leukemia, impaired cellular immunity, HIV infections and several others (Brett & Woods, 2000). Moreover, infection of melioidosis can be asymptomatic in patients as the disease is able to stay dormant for 29 years up to 62 years (Inglis *et al.*, 2006; Galyov *et al.*, 2010).

The genomic DNA of *B. pseudomallei* is comprised of two circular chromosomal DNA that reach up to 7.2 Mb in size which consisted of more than 5,600 protein-coding genes and a vast collection of virulence genes that encode for adhesins, toxins, clusters of capsular polysaccharide, and diverse secretion systems of type III and IV (Nandi & Tan, 2013). The larger chromosome among the two typically associated with the bacterial growth and metabolism while the smaller chromosome is responsible to the adaptation of the bacterium to various environmental conditions (Inglis *et al.*, 2006). According to Sarovich *et al.* (2014), there were several virulence factors which had been identified through previous research studies on *B. pseudomallei* such as Bsa type III secretion system cluster 3, cytotoxin Burkholderia lethal factor 1 and a few of others yet the severity of

melioidosis and its vast spectrum of presentations may also be influenced by diverse virulence factors that are not yet discovered and identified.

2.2. Previous studies on *Burkholderia* species and *Burkholderia* closely related sp. tested in the project

2.2.1. *Burkholderia cepacia*

B. cepacia is classified under *Burkholderia cepacia-complex* (BCC) group which is comprised of 9 different genomovars including *Burkholderia pyrrocinia* and *Burkholderia multivorans* (Matthaiou *et al.*, 2011). It is identified as a Gram-negative, aerobic, motile bacillus which displays a wide range of nutritional versatility (Matthaiou *et al.*, 2010). The species is considered as an opportunistic pathogen as it is capable of causing infections in hospitalized and immunocompromised patients as well as drug addicts with chronic granulomatous disease and cystic fibrosis (CF) (Matthaiou *et al.*, 2011). There are many experimental studies conducted which indicate the ability of *B. cepacia* to exhibit antibiosis activity against many species of fungi and streptomycetes (El-Banna & Winkelmann, 1998) as well as root-knot nematode, *Meloidogyne incognita* (Meyer *et al.*, 2000). However, the study on the presence of antibiosis activity against *B. pseudomallei* by *B. cepacia* is still unclear and lack in information.

2.2.2. *Burkholderia humptydooensis*

B. humtydooensis was first described by Gee *et al.* (2008) as *Burkholderia thailandensis*-like strain after carrying out DNA-DNA hybridization to distinguish the strain from *B.*